Supplementary Material

Methods

Supplementary Table 1

Primer Name	**Primer Sequence**
S248F Forw.	5’ GCATC**TTC**GTGCTGCTTTCTCTCACCGTCTTCCTGCTGC 3’
S248F Rev.	5’ GCAC**CGA**GATGCACAGCGTGACCTTCTCGCCGCACTCC 3’
S252L Forw.	5’ GCTT**TTG**CTCACCGTCTTCCTGCTGCTCATCACCGAG 3’
S252L Rev.	5’ CGGTGAG**CAA**AAGCAGCACCGAGATGCACAGCGTGACC 3’
776ins3 Forw.	5’ GCTC**CTG**ATCACCGAGATCATCCCGTCCACCTCGCTGG 3’
776ins3 Rev.	5’ CGGTGAT**CAG**GAGCAGCAGGAAGACGGTGAGAGAAAGC 3’
V287L Forw.	5’ CCAAGATT**CTG**CCTCCCACCTCCCTCGACGTACCGCTGG 3’
V287L Rev.	5’ GGGAGG**CAG**AATCTTGGAGATGAGCAGCAGGAACACCG 3’
V287M Forw.	5’ CCAAGATT**ATG**CCTCCCACCTCCCTCGACGTACCGCTGG 3’
V287M Rev.	5’ GGGAGG**CAT**AATCTTGGAGATGAGCAGCAGGAACACCG 3’

Fluorescence Intensity Ratio Determinations of Subunit Stoichiometry

Fluorescence intensity ratio (FIR) analysis provides information to define the subunit stoichiometry of most heteromeric channel types. The method is based on fluorescently tagged subunits and was first reported (Zheng and Zagotta, 2004) to determine the subunit stoichiometry of olfactory cyclic nucleotide-gated channels. Our studies used the same YFP- and CFP-tagged α4 and β2 subunits used in the FRET studies. However, whereas the FRET studies employed 1:1 ratios of α4CFP:α4YFP (or the β2 equivalents), the FIR studies used two sets of cDNAs: (1) mixtures of α4CFP and β2YFP cDNAs, or (2) α4YFP and β2CFP cDNAs. Since the channel subunit and the fluorescent protein are covalently linked, the molar ratio between CFP and YFP molecules is the same as the molar ratio between the subunits in which they are inserted. To correct for different excitation laser intensities and different extinction coefficients and quantum yields of the fluorophores, a similar measurement was carried with set 1 and set 2. By comparing the two fluorescence ratios, we calculate the correction factor to account for the different intensities of the individual fluorophores and thereby calculate the ratio of subunits.

There are potential concerns with the FIR method. (a) FRET may occur between channel subunits. FIR assumes that fluorescence emission of CFP and YFP are independent. Considering the close proximity of channel subunits, this assumption is not true in most cases due to FRET between these fluorophores. (b) There may unassembled subunits present. (c) There may be degraded subunits, producing soluble CFP and YFP. Points (b) and (c) Would contribute to the fluorescence intensities measured, thus obscuring the subunit ratios calculated for the assembled channels. To overcome these complications, we used an analysis that we term “FRET-defined

FIR" to calculate the nAChR subunit stoichiometry. FRET-defined FIR assumes that FRET occurs only in fully assembled receptors, and that partially assembled receptors, free subunits, or free fluorophores do not contribute appreciable FRET.

For simplicity, we define α = α4 and β = β2. When CFP-tagged α subunits and YFP-tagged β subunits are co-expressed, the intensities of CFP and YFP can be calculated as $F_{CFP} = C_1[\alpha]$ and $F_{YFP} = C_2[\beta]$, where F_{CFP} and F_{YFP} are CFP and YFP intensities calculated by acceptor photobleaching. Thus, F_{CFP} corresponds to the dequenched CFP intensity when 100% of the acceptor molecules are bleached; this represents CFP carrying subunits participating in assembled pentamers with YFP containing subunits. Similarly F_{YFP} detected by exciting CFP at 439 nm and detecting the YFP emission due to FRET, arises from YFP containing subunits participating in assembled pentamers with CFP containing subunits. Both intensities were detected by spectral imaging and unmixed to eliminate background fluorescence and the overlap of emission spectra. The [α] and [β] are the number of αCFP and βYFP subunits. The constants C_1 and C_2 include the laser intensities, the system transfer function, the properties of the fluorophores, and other factors. But C_1 and C_2 are independent of the subunit (α vs β) hosting the fluorophore. The FIR

$$k_1 = \frac{F_{CFP}}{F_{YFP}} = C\frac{[\alpha]}{[\beta]}, \qquad \text{(Equation 10)}$$

where $C = C_1 / C_2$. Similarly, coexpressing αYFP and βCFP subunits yields an FIR

$$k_2 = \frac{F_{CFP}}{F_{YFP}} = C\frac{[\beta]}{[\alpha]}. \qquad \text{(Equation 11)}$$

Therefore, both the subunit ratio and the parameter *C* were determined using the following equations:

$$\frac{[\alpha]}{[\beta]} = \sqrt{k_1 / k_2}\,; \qquad \text{(Equation 12)}$$

$$C = \sqrt{k_1 k_2} \qquad \text{(Equation 13)}$$

Once *C* was experimentally determined for our optical system, we calculated k_1 and k_2 for any given subunit ratio. Comparison of the experimental data with these calculated values revealed the subunit stoichiometry under the experimental conditions.

(Supplementary) Figure 1. FIR measurements of the subunit ratio for α4β2 nAChRs. Scatter plot of the CFP intensity versus the YFP intensity (arbitrary calibrated units, (ACU)), measured from channels formed by α4YFP and β2CFP subunits (A, C and E), or α4CFP β2YFP (B, D and F). Each point is from an individual N2a cell. The dotted lines represent predicted fluorescence intensity ratio for 100% $(\alpha4)_2(\beta2)_3$ stoichiometry and the dashed lines represent 100% $(\alpha4)_3(\beta2)_2$ stoichiometry; the black lines are linear fits to each data set.

A, B, 1:1 ratio of α4:β2 cDNA.

C, D, 1:4 ratio of α4:β2 cDNA.

E, F, 1:9 ratio of α4:β2 cDNA.